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Hypermethylated *RASSF1A* in Maternal Plasma: A Universal Fetal DNA Marker that Improves the Reliability of Noninvasive Prenatal Diagnosis

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Background: **We recently demonstrated that the promoter of the *RASSF1A* gene is hypermethylated in the placenta and hypomethylated in maternal blood cells. This methylation pattern allows the use of methylation-sensitive restriction enzyme digestion for detecting the placental-derived hypermethylated *RASSF1A* sequences in maternal plasma.**

Methods: **We performed real-time PCR after methylation-sensitive restriction enzyme digestion to detect placental-derived *RASSF1A* sequences in the plasma of 28 1st-trimester and 43 3rd-trimester pregnant women. We used maternal plasma to perform prenatal fetal rhesus D (RhD) blood group typing for 54 early-gestation RhD-negative women, with hypermethylated *RASSF1A* as the positive control for fetal DNA detection.**

Results: **Hypermethylated *RASSF1A* sequences were detectable in the plasma of all 71 pregnant women. The genotype of plasma *RASSF1A* after enzyme digestion was identical to the fetal genotype in each case, thus confirming its fetal origin. Nineteen of the 54 pregnant women undergoing prenatal fetal RhD genotyping showed undetectable *RHD* sequences in their plasma DNA samples. The fetal DNA control, *RASSF1A*, was not detectable in 4 of the 19 women. Subsequent chorionic villus sample analysis revealed that 2 of these 4 women with negative *RHD* and *RASSF1A* signals were in fact carrying RhD-positive fetuses.**

Conclusions: **Hypermethylated *RASSF1A* is a universal marker for fetal DNA and is readily detectable in maternal plasma. When applied to prenatal RhD genotyping, this marker allows the detection of false-negative results caused by low fetal DNA concentrations in maternal plasma. This new marker can also be applied to many other prenatal diagnostic and monitoring scenarios.**

Circulating fetal DNA analysis in maternal plasma is useful in the prenatal diagnosis of sex-linked disorders *(1)*, fetal rhesus D (RhD)[6] status *(2)*, and β-thalassemia *(3)*. Many of these applications focus on the detection of paternally inherited disease-causing sequences in maternal plasma. Whether the fetus has inherited such a sequence is inferred by the presence or absence of the target sequence in maternal plasma, but the absence of such a sequence in maternal plasma could alternatively be a result of low circulating fetal DNA concentrations or fetal DNA loss during sample processing. Thus, the availability of a positive control confirming the presence of fetal DNA in the sample would be a useful analytical safeguard measure *(4)*. In addition, the quantitative measurement of a fetal DNA target that could be similarly detected in pregnancies with male or female fetuses is useful for monitoring and predicting pregnancy-related conditions associated with aberrant fetal DNA concentrations, including preeclampsia *(5–7)* and certain fetal chromosomal aneuploidies *(8, 9)*.

Existing fetal DNA markers, such as Y-chromosomal sequences, cannot serve adequately as positive controls

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[6] Nonstandard abbreviations: RhD, rhesus D; CVS, chorionic villus sample.

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because they are applicable only to pregnancies carrying a male fetus. To develop a positive control system for fetal DNA detection, genetic variations that differ between the mother and the fetus could be used. Such genetic variation–based approaches, however, would require the use of multiple markers to ensure that at least one would be informative and therefore may increase the complexity of the analysis *(10)*. Alternatively, an epigenetic approach could be explored *(11)*. We recently reported that the *maspin* (*SERPINB5*)[7] gene promoter was heavily methylated in maternal blood cells and hypomethylated in the placenta *(11)*. We showed that placental-derived hypomethylated *maspin* was detectable in maternal plasma regardless of the fetal sex and genetic variations and thus could serve as a universal fetal DNA marker *(11)*. Operationally, the detection of unmethylated *maspin* sequences among a background of methylated maternal sequences requires the relatively tedious and labor-intensive procedure of bisulfite conversion *(11)*. Bisulfite treatment of a DNA sample could also lead to DNA degradation of up to 96% *(12)*, thus greatly decreasing the ease and efficiency of the use of hypomethylated *maspin* detection as a positive control for fetal DNA in a routine clinical setting.

In search of other fetal epigenetic markers, we recently demonstrated that the promoter of the *RASSF1A* tumor suppressor gene is hypermethylated in the placenta but hypomethylated in maternal blood cells, a methylation pattern that is exactly opposite that of the *maspin* promoter (unpublished data). Consequently, the background maternally derived hypomethylated *RASSF1A* sequences could potentially be removed by methylation-sensitive restriction enzyme digestion, whereas the hypermethylated placental (fetal) *RASSF1A* sequences are expected to be resistant to methylation-sensitive restriction enzyme digestion and thereby should be detectable and quantifiable by real-time PCR.

To illustrate the clinical application of such a universal fetal DNA positive control in noninvasive prenatal diagnosis, we incorporated the use of this new *RASSF1A* system and the existing *SRY* system *(13)* as positive controls for fetal DNA to perform prenatal RhD blood group typing for a cohort of pregnant women. Our aim was to investigate whether the new *RASSF1A* system would be more effective than the existing *SRY* system for detecting cases falsely negative for *RHD*.

Materials and Methods

PATIENT RECRUITMENT AND SAMPLE COLLECTION

For the module investigating the noninvasive prenatal detection of RhD blood group, pregnant women were recruited from the King's College Hospital, London, United Kingdom. These patients were undergoing 1st-trimester Down syndrome screening, but all fetuses were later confirmed to be karyotypically normal. For other modules, patients were recruited from the Department of Obstetrics and Gynaecology, Prince of Wales Hospital, Hong Kong. The study was approved by the institutional review boards of the participating clinical units. Informed consent was obtained from all participating women.

Chorionic villus samples (CVSs) were collected primarily for Down syndrome prenatal diagnosis. Third-trimester placental tissues were collected after elective cesarean delivery of uncomplicated pregnancies. Maternal peripheral blood samples were collected just before the performance of obstetric procedures and at 24 h after delivery.

SAMPLE PROCESSING AND DNA DIGESTION BY METHYLATION-SENSITIVE RESTRICTION ENZYME

Placental tissue and blood cells. DNA was extracted from 200 μL of maternal blood cells and 0.2 g of placental tissue with the QIAamp DNA Blood Minikit (Qiagen) and the QIAamp DNA Minikit (Qiagen), respectively, and 100 ng of placental and maternal blood cell DNA were digested with 100 U of *Bst*U I, a methylation-sensitive restriction enzyme, at 60 °C for 16 h.

Plasma samples. Plasma was harvested *(14)* and DNA was extracted from 800 μL (RhD blood typing module) or 1.6 mL (other modules) of plasma with the QIAamp Minikit and eluted with 50 μL of H_2O, and 35 μL of plasma DNA were digested with 100 U of *Bst*U I enzyme at 60 °C for 16 h.

BISULFITE SEQUENCING OF THE *BETA-ACTIN* GENE IN PLACENTA AND MATERNAL BLOOD CELLS

We bisulfite-converted 1 μg of each extracted DNA sample with the CpGenome Universal DNA Modification Kit (Chemicon) according to the manufacturer's recommendations. Each bisulfite-converted DNA sample was subjected to PCR amplification with the primers 5′-TTATTTGGYGGYGGGTGTGGA-3′ and 5′-TCCCTCCT-CCTCTTCCTCAATCTC-3′. The PCR product was cloned and sequenced. After confirming the completeness of bisulfite conversion, the CpG sites sequenced as cytosine or thymine residues were scored as methylated or unmethylated, respectively.

REAL-TIME DETECTION OF *SRY*, *RASSF1A*, AND *BETA-ACTIN* SEQUENCES

Real-time PCR assays were established for the detection of *SRY*, *RASSF1A*, and *beta-actin* (*ACTB*) sequences. The sequences of the primers and probes are listed in Table 1. The contents and thermal profiles of the 3 assays were identical apart from the primers and probes used. Each reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nmol/L each primers, and 85 nmol/L probes. We used 5 μL of non–enzyme-treated plasma DNA or 7.15 μL of enzyme-digested plasma DNA mixture (equivalent to 5 μL of undigested plasma DNA)

[7] Human genes: *maspin* (*SERPINB5*), serpin peptidase inhibitor, clade B (ovalbumin), member 5; *beta-actin* (*ACTB*), actin, beta; *RASSF1A*, Ras association (RalGDS/AF-6) domain family 1A; *RHD*, Rh blood group, D antigen.

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Table 1. Primers and probes for the real-time PCRs for *RHD*, *RASSF1A*, *beta-actin*, and *SRY*.

Target	Name	Sequence	Primer/probe
RASSF1A	RSF-b151F	5′-AGC CTG AGC TCA TTG AGC TG-3′	Primer
RASSF1A	RSF-dsgnR	5′-ACC AGC TGC CGT GTG G-3′	Primer
RASSF1A	RSF-dsgnT	5′-FAM-CCA ACG CGC TGC GCA T(MGB)-3′[a]	Probe
beta-actin	Actin-163F	5′-GCG CCG TTC CGA AAG TT-3′	Primer
beta-actin	Actin-298R	5′-CGG CGG ATC GGC AAA-3′	Primer
beta-actin	Actin-243T	5′-FAM-ACC GCC GAG ACC GCG TC(MGB)-3′	Probe
SRY	SRY-109F	5′-TGG CGA TTA AGT CAA ATT CGC-3′	Primer
SRY	SRY-245R	5′-CCC CCT AGT ACC CTG ACA ATG TAT T-3′	Primer
SRY	SRY-142T	5′-FAM-AGCAGT AGA GCA GTC AGG GAG GCA GA(TAMRA)-3′	Probe

[a] MGB, minor-groove binding.

as the template for each PCR. The thermal profile was 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. All reactions were run in duplicate, and the mean quantity was taken. A DNA construct containing 1 copy each of the *RASSF1A*, *SRY*, and *beta-actin* amplicons was established as the quantitative standard of the 3 assays. The detection limit for all the 3 assays was 1 copy per reaction.

ENZYME DIGESTION EFFICIENCIES FOR UNMETHYLATED *RASSF1A* AND *BETA-ACTIN* SEQUENCES

To investigate the similarity of the enzyme digestion efficiencies for unmethylated *RASSF1A* and *beta-actin* sequences, 1-μg aliquots of maternal buffy coat were digested with 100 U of *Bst*U I enzyme for different time intervals (from 15 min to 16 h). The concentrations of *RASSF1A* and *beta-actin* sequences were measured in each sample after the enzyme digestion.

REAL-TIME DETECTION OF *RHD* SEQUENCES

The RhD genotype was determined by real-time amplification of 2 regions located in exon 7 and exon 10 of the *RHD* gene as previously described (*2*, *15*). The results of the 2 assays were identical in all cases. Therefore, the results in the subsequent sections will be presented as *RHD*-positive or *RHD*-negative only.

DUPLEX REAL-TIME PCR ASSAYS FOR *RASSF1A* AND *BETA-ACTIN*

A duplex assay for the simultaneous amplification of *RASSF1A* and *beta-actin* was developed for the RhD blood group typing module. The reaction mix, thermal profile, primers, and probes used in this assay were identical to those used in the monoplex assays, apart from changing the fluorescent reporter dye of the *beta-actin* TaqMan probe to VIC™ (Applied Biosystems). The final concentrations of the *beta-actin* primers and probe were increased to 450 nmol/L and 126 nmol/L, respectively.

GENOTYPING OF THE *RASSF1A* GENE

A single nucleotide polymorphism (id rs4688725) is located within the *RASSF1A* amplicon. We determined *RASSF1A* genotypes for maternal blood cells, the placenta, and maternal plasma DNA with and without *Bst*U I enzyme digestion. PCR amplification of the *RASSF1A* sequence was performed with the primers RSF-b151F and RSF-dsgnR. After PCR amplification, we set up a primer extension reaction, based on the homogeneous MassEXTEND protocol (Sequenom), and each 14-μL reaction contained 10 μL of PCR products, 0.77 μmol/L extension primer Rsf-R17 5′-CAG CCG GGT GGG CCC T-3′, 1.15 U of thermosequenase, and a mixture of dideoxynucleotides (ddATP, ddCTP, and ddTTP) and the deoxynucleotide dGTP (64 μmol/L each). For a *RASSF1A* sequence with an allelotype A, the primer would be extended to produce 5′-CAG CCG GGT GGG CCC TddT-3′ with a molecular mass of 5476.6 Da. For a *RASSF1A* sequence with an allelotype C, the primer would be extended to produce 5′-CAG CCG GGT GGG CCC TGd dC-3′ with a molecular mass of 5790.8 Da. The final base extension products were analyzed by the MassARRAY compact matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Sequenom). The genotype of the *RASSF1A* was determined by the TyperAnalyzer software (Sequenom).

Results

DETECTION OF *RASSF1A* AND *BETA-ACTIN* SEQUENCES IN PLACENTAL TISSUES AND MATERNAL BLOOD CELLS WITH AND WITHOUT *Bst*U I ENZYME DIGESTION

Because *Bst*U I is a methylation-sensitive restriction enzyme, we would expect hypomethylated DNA sequences, such as the *RASSF1A* molecules derived from maternal blood cells, to be digestible and not detectable after enzyme digestion. Without enzyme digestion, *RASSF1A* sequences were detectable in both the placental tissues and maternal blood cells. After enzyme digestion, only *RASSF1A* molecules from the placenta were detected. In contrast, *beta-actin* molecules were detectable only without enzyme digestion, regardless of their origins. This finding is in line with the bisulfite sequencing results that all CpG sites in the *beta-actin* amplicon were unmethylated in both the placenta and the maternal blood cells (see Fig. 1 in the Data Supplement that accompanies the online

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version of this article at http://www.clinchem.org/content/vol52/issue12).

COMPARISON OF THE ENZYME DIGESTION EFFICIENCIES FOR UNMETHYLATED *RASSF1A* AND *BETA-ACTIN* SEQUENCES

The concentrations of *RASSF1A* and *beta-actin* sequences showed a positive correlation for any given extent of incomplete enzyme digestion (r = 0.986; P <0.0001; Pearson correlation; see Fig. 2 in the online Data Supplement). These data suggest that the enzyme digestion efficiencies for unmethylated *RASSF1A* and *beta-actin* sequences were similar, thus justifying the use of *beta-actin* as a control for the completeness of the enzyme digestion of unmethylated *RASSF1A* sequences.

DETECTION OF *RASSF1A* IN MATERNAL PLASMA AFTER ENZYME DIGESTION

We analyzed the plasma samples of 71 pregnant women (28 in the 1st trimester and 43 in 3rd trimester) and 25 nonpregnant women for *RASSF1A* after enzyme digestion. The median fetal gestational ages for 1st-trimester and 3rd-trimester pregnant women were 12 weeks and 38 weeks, respectively. *RASSF1A* sequences were detectable in all pregnant women but not in those who were not pregnant (Fig. 1). The median plasma *RASSF1A* concentrations of the 1st-trimester and 3rd-trimester pregnant women were 40 copies/mL and 245 copies/mL, respectively. The *beta-actin* digestion control was analyzed for all the samples. In 4 plasma samples, *beta-actin* sequences were detectable after enzyme digestion (3 from pregnant women and 1 from a nonpregnant woman). There was no statistically significant difference in the rate of incomplete enzyme digestion between the pregnant and nonpregnant women (4.2% vs 4.0%; P = 1.0; Fisher exact test).

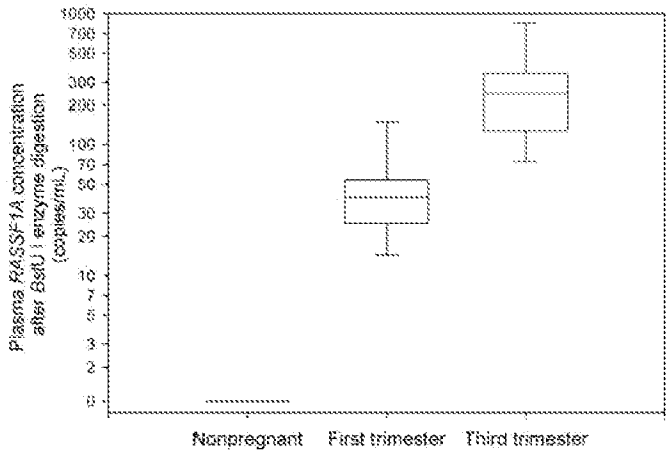

Fig. 1. Box plot of the plasma concentrations of *RASSF1A* sequence after *Bst*U I enzyme digestion in 25 nonpregnant women and 28 1st-trimester and 43 3rd-trimester pregnant women.

The *upper* and *lower whiskers* represent the 90th and 10th percentiles, respectively. The *upper*, *middle*, and *lower bars* of the *box* represent the 75th, 50th, and 25th percentiles, respectively. The *y-axis* is in logarithmic scale.

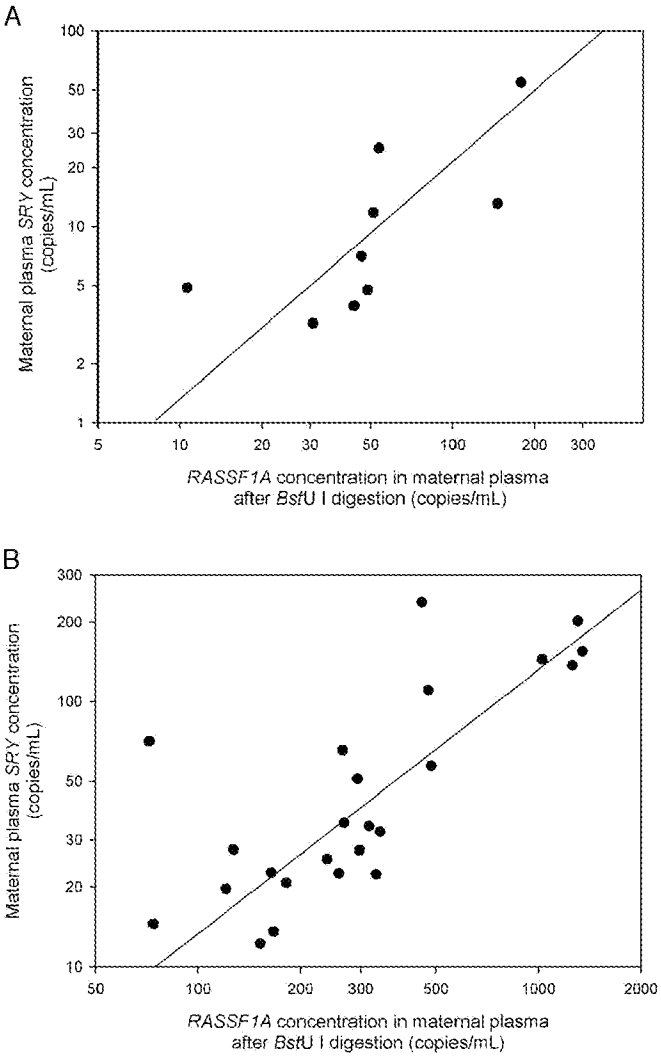

Fig. 2. Correlation of the plasma concentrations of the enzyme-digestion–resistant *RASSF1A* and the *SRY* sequences in the 9 1st-trimester (*A*) and the 24 3rd-trimester (*B*) pregnant women carrying male fetuses.

The *RASSF1A* concentration after enzyme digestion shows a positive correlation with *SRY* concentration for the 1st-trimester (r = 0.850; P <0.0001; Spearman correlation) and the 3rd-trimester (r = 0.717; P <0.0001; Spearman correlation) pregnancies. The *x-axis* and *y-axis* represent the plasma concentrations of the *RASSF1A* and *SRY* sequences, respectively, and both *axes* are in logarithmic scale.

The enzyme digestion was repeated in these cases, with *RASSF1A* and *beta-actin* subsequently becoming undetectable.

CLEARANCE OF HYPERMETHYLATED *RASSF1A* SEQUENCE IN MATERNAL PLASMA AFTER DELIVERY

Five pregnant women each carrying a male fetus were recruited for the study of the postdelivery clearance of *RASSF1A* sequences in enzyme-digested maternal plasma. Both *RASSF1A* and *SRY* sequences were detectable in the plasma before delivery but were completely cleared at 24 h after delivery.

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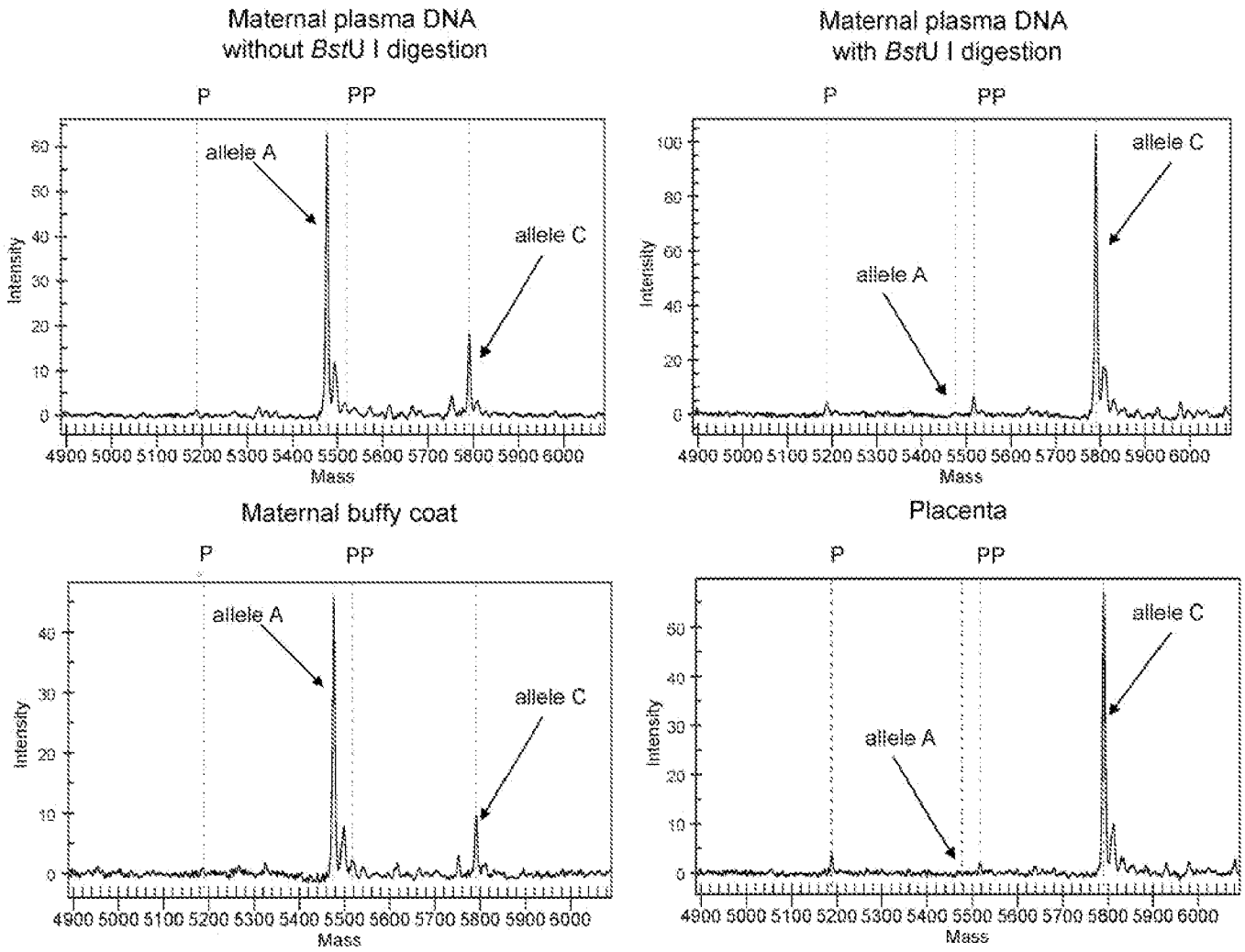

Fig. 3. The genotyping results of *RASSF1A* for the plasma DNA (with and without *BstU* I enzyme digestion), maternal blood cells, and placenta of a pregnant woman.

In each mass spectrum, the *x-axis* represents the mass of the ions and the *y-axis* represents the ion current detected by the mass spectrometer in arbitrary units. The peak corresponding to the mass 5476.6 Da represents the A allele, and the peak corresponding to the mass 5790.8 Da represents the C allele. *P* and *PP* represent the unextended primers and pausing products, respectively. The pausing products are the result of premature termination of the base extension reaction after an incorporation of a dGTP for the C allele DNA template. The peaks corresponding to the A and C alleles are present in the mass spectra for the undigested plasma DNA and maternal blood cells. Thus, the genotypes of both the undigested plasma DNA and maternal blood cells are AC. For the enzyme-digested plasma DNA and placenta, only the peak corresponding to the C allele was observed. Hence, the genotypes of both the enzyme-digested plasma DNA and placenta are CC.

POSITIVE CORRELATION BETWEEN THE PLASMA CONCENTRATIONS OF *SRY* AND ENZYME-DIGESTION–RESISTANT *RASSF1A* SEQUENCES

The plasma concentrations of *SRY* and *RASSF1A* (with and without enzyme digestion) were studied in the 9 1st-trimester and the 24 3rd-trimester pregnant women, each carrying a single male fetus. A positive correlation was observed between the plasma concentrations of enzyme-digestion–resistant *RASSF1A* and *SRY* sequences for the 1st-trimester ($r = 0.850$; $P < 0.0001$; Spearman correlation; Fig. 2A) and the 3rd-trimester ($r = 0.717$; $P < 0.0001$; Spearman correlation; Fig. 2B) pregnant women. Because the only source of *SRY* in maternal plasma was the fetal tissues, this observation further supported the fetal origin of enzyme-digestion–resistant *RASSF1A*. In contrast, the concentration of *RASSF1A* without enzyme digestion, which reflected the concentration of molecules predominantly derived from the mother, did not correlate with the concentration of *SRY* for the 1st-trimester ($r = 0.083$; $P = 0.81$; Spearman correlation) and the 3rd-trimester ($r = 0.228$; $P = 0.28$; Spearman correlation) pregnant women.

IDENTICAL *RASSF1A* GENOTYPES FOR THE ENZYME-DIGESTED PLASMA AND PLACENTA

The *RASSF1A* genotypes of the fetus (placentas or CVSs), mother (maternal blood cells), and enzyme-digested maternal plasma DNA were determined for 43 3rd-trimester (also with genotypes of undigested plasma DNA) and 16 1st-trimester pregnant women. The genotyping mass

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spectra of a typical case (Fig. 3) show that the genotype of the maternal plasma DNA without enzyme digestion (AC) was identical to that of the maternal blood cells (AC). This is because the total circulating *RASSF1A* sequences were predominantly maternally derived. On the other hand, the genotype of the maternal plasma DNA after enzyme digestion (CC) was the same as the placental genotype (CC). This finding suggests that circulating enzyme-digestion–resistant *RASSF1A* sequences were derived from fetal tissues. The *RASSF1A* genotyping results of 59 pregnant women (16 1st-trimester and 43 3rd trimester) (Table 2) show that in each of the 59 cases, the genotype of the maternal plasma DNA after enzyme digestion was identical to the placental (fetal) genotype.

Table 2. Genotype assessment of *RASSF1A* sequences in maternal blood cells, placenta (or CVS), and plasma DNA with and without (only for 3rd-trimester cases) methylation-sensitive restriction enzyme digestion.[a]

A. 3rd-trimester pregnant women.

	Genotype			
No. of cases[b]	Blood cells	Undigested plasma	Placenta	Digested plasma
5	AA	AA	AA	AA
11	AC	AC	AC	AC
12	CC	CC	CC	CC
3	**AA**	**AA[c]**	**AC**	**AC**
3	**AC**	**AC**	**AA**	**AA**
7	**AC**	**AC**	**CC**	**CC**
2	**CC**	**CC[c]**	**AC**	**AC**

B. 1st-trimester pregnant women.

	Genotype		
No of cases[b]	Blood cells	CVS	Digested plasma
3	AC	AC	AC
6	CC	CC	CC
1	**AA**	**AC**	**AC**
3	**AC**	**AA**	**AA**
3	**AC**	**CC**	**CC**

[a] Situations in which the genotypes of the blood cells (mother) are different from those of the placenta (fetus) are highlighted in **boldface**. In each of these scenarios, the genotypes of the enzyme-digested plasma DNA are identical to those of the placenta or CVS.

[b] The number of pregnant women showing that particular genotype combination for the blood cells, placenta, and plasma.

[c] The fetal-specific allele is not detectable in the undigested plasma of these cases because the fractional concentration of the fetal sequences is below the detection limit of the mass spectrometry system. In the 3rd trimester, fetal DNA constitutes a mean of 6% of the total cell-free DNA in maternal plasma. This implies that each allele of a heterozygous fetus constitutes only a mean of 3% of the total circulating *RASSF1A* sequences in the maternal plasma of a homozygous mother. By mixing buffy coat DNA from individuals with genotypes AA and CC in different proportions, we have shown that this *RASSF1A* genotyping assay is able to detect a minor allele only when it constitutes ≥10% of the total *RASSF1A* sequences.

NONINVASIVE PRENATAL RHD BLOOD TYPING WITH ENZYME-DIGESTION–RESISTANT *RASSF1A* AS A POSITIVE CONTROL FOR FETAL DNA

Of 355 pregnant women screened for RhD status, 54 were RhD-negative, and their plasma and CVS were subjected to further investigation for fetal RhD status. *RHD* sequences were detected in the maternal plasma of 35 of these 54 pregnant women. In the plasma DNA samples of the remaining 19 women with negative maternal plasma *RHD* results, 15 were positive for enzyme-digestion–resistant *RASSF1A* sequences and the other 4 showed negative results. In the former group, the positive detection of the *RASSF1A* sequences in maternal plasma confirmed the presence of fetal DNA, and therefore the negative *RHD* results could be confidently interpreted. Indeed, CVS analysis revealed that all these 15 women were carrying RhD-negative fetuses. On the contrary, the failure to detect *RASSF1A* sequences in the remaining 4 cases suggested that their negative *RHD* results should be interpreted with caution. In fact, the CVSs were positive for *RHD* in 2 of these 4 cases, indicating that the maternal plasma *RHD* results were falsely negative because of low fetal DNA concentrations in those plasma samples. These results indicate that the *RASSF1A* assay has served its function in flagging potentially problematic cases for further investigation. Of the 19 plasma samples negative for *RHD*, only 6 were positive for *SRY*, thus highlighting the inadequacies of *SRY* as an internal control. The flow chart of the screening procedures and results is presented in Fig. 4.

Discussion

In this study, we developed a new test for the detection of fetal DNA in maternal plasma. This test is based on the detection of a hypermethylated placental (fetal) DNA sequence in the maternal circulation. In contrast to existing fetal DNA markers that detect Y-chromosomal sequences and genetic variations between the fetus and mother, this new method is applicable to all pregnancies irrespective of the sex and genetic variations of the fetus.

The methylation pattern of the *RASSF1A* promoter in the placenta and maternal blood cells allows the use of methylation-sensitive restriction enzyme digestion for specifically cutting the maternally derived background *RASSF1A* sequences while leaving the placentally (fetal) derived *RASSF1A* sequences intact. This system is expected to be more sensitive, more reliable, and easier to use than the bisulfite-based method used for the detection of unmethylated *maspin* sequences *(11)*. In this study, *RASSF1A* sequences were detectable in all the 1st- and 3rd-trimester maternal plasma samples after enzyme digestion.

In addition, an internal control system was devised for the detection of incomplete enzyme digestion, which could potentially lead to false-positive results. This internal control system consisted of a real-time PCR assay targeting the *beta-actin* gene promoter. Because, as we

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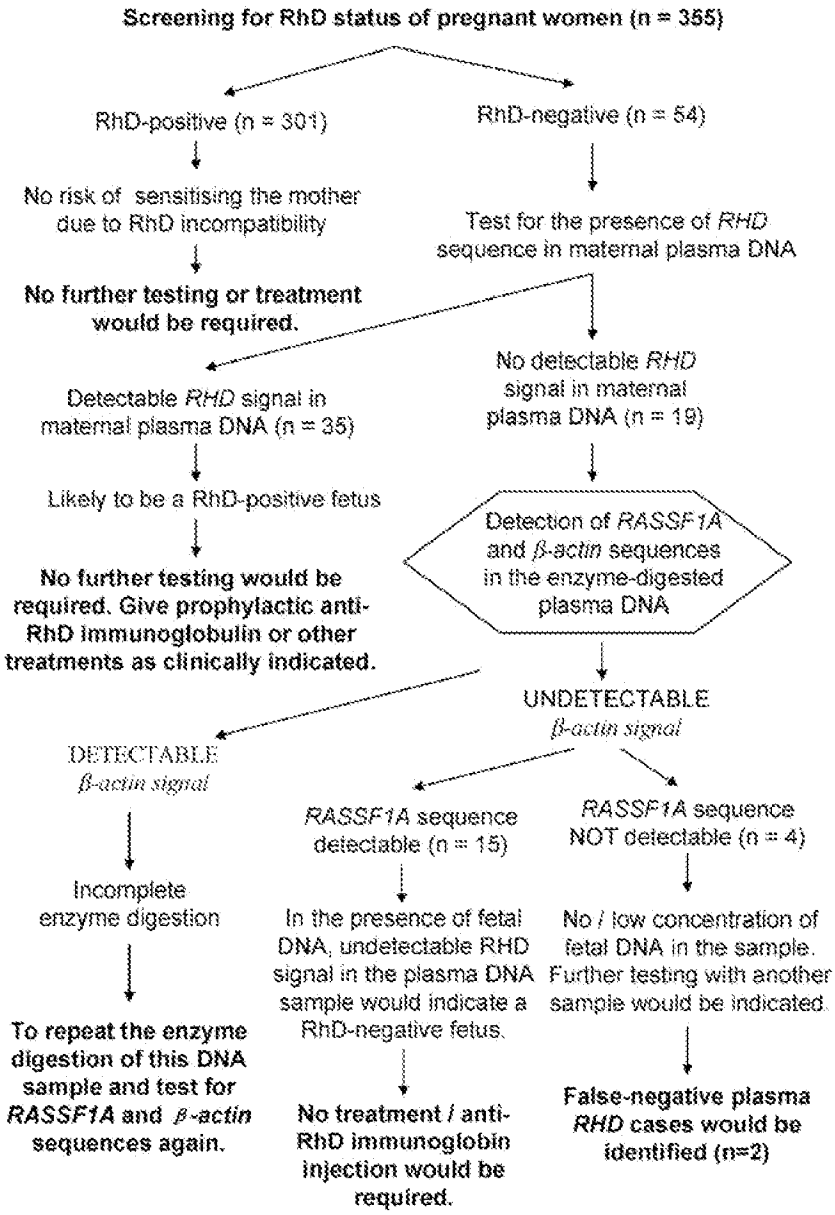

Fig. 4. A schematic diagram showing the logistics and the results of the prenatal screening for potential fetal RhD incompatibility among 355 pregnant women.

The number of women in each category is in *brackets*.

have shown, the digestion efficiencies of unmethylated *beta-actin* and *RASSF1A* sequences are similar, an undetectable *beta-actin* signal should reflect the completeness of the enzyme digestion of unmethylated *RASSF1A* sequences. During the evaluation of the detectability of *RASSF1A* sequences in enzyme-digested plasma DNA, this digestion control system detected 4 cases of incomplete enzyme digestion. By duplexing the *RASSF1A* and *beta-actin* assays, the robustness of this new fetal DNA test is further enhanced.

Our findings demonstrate that the enzyme-digestion–resistant *RASSF1A* sequences that are detectable in maternal plasma are of fetal origin because (*a*) there was postpartum clearance of such sequences; (*b*) they were not detectable in the plasma of nonpregnant women; (*c*) a positive correlation between the maternal plasma concentration of enzyme-digestion–resistant *RASSF1A* and *SRY* sequences was observed, reaffirming their common fetal origin; and (*d*) in every case the plasma *RASSF1A* genotype was identical to the fetal genotype after enzyme digestion.

In addition to being a potentially useful generic circulating fetal DNA marker for the monitoring and assessment of pregnancy-related conditions, the new test was an ideal system to be used as a positive control for fetal DNA detection in maternal plasma. Hence, in the last part of the study, we attempted to demonstrate the clinical utility of such a positive control system in prenatal diagnosis. We recruited patients from early gestation and used a reduced volume of plasma for DNA extraction. As a result, some of the extracted plasma samples would be expected to have very low fetal DNA concentrations. Of

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the 19 RhD-negative pregnant women with no detectable *RHD* sequence in their plasma, 15 were positive for *RASSF1A*, thus confirming the presence of fetal DNA in these plasma DNA samples. Therefore, the negative detection of *RHD* in their plasma should indicate a RhD-negative fetus. For the 4 cases that were negative for both *RHD* and enzyme-digestion–resistant *RASSF1A*, their fetal RhD typing results should be regarded as inconclusive because of the absence or low concentration of fetal DNA in their plasma samples. Through CVS analysis, 2 of these 4 cases were confirmed to be carrying RhD-positive fetuses. Thus, for these 2 cases, their falsely negative maternal plasma *RHD* results were correctly identified by our failure to detect the positive control, enzyme-digestion–resistant *RASSF1A*. Although the CVSs of the other 2 cases were negative for *RHD*, the absence of *RHD* signal could be due either to a low fetal DNA concentration in their plasma DNA samples or an absence of *RHD* sequence in the fetus. In other words, these 2 apparently correct RhD typing results could merely be a coincidence of the collection of maternal plasma samples with low fetal DNA concentration from pregnant women carrying RhD-negative fetuses. In real-life situations in which no CVSs would be available, any fetal *RHD* typing results with negative detection of enzyme-digestion–resistant *RASSF1A* should be regarded as inconclusive, and further testing with another plasma sample would be indicated.

To further illustrate the advantage of using a sex-independent fetal marker, the results were compared with an existing fetal DNA marker, *SRY*. The *SRY* assay would be positive only when the fetus is a male. Of the 19 women with negative detection of *RHD* sequence in their plasma, only 6 were positive for *SRY*, which further confirmed an RhD-negative fetus. In the remaining 13 cases, whether the negative detection of *RHD* and *SRY* sequences in the plasma DNA was a result of a female RhD-negative fetus or the inadequate fetal DNA in the maternal plasma could not be ascertained.

In this study, we developed a universal fetal DNA marker that can be readily detected in maternal plasma. As illustrated in the example of noninvasive fetal RhD status determination, this new test is invaluable in prenatal diagnostic procedures as a positive fetal DNA control. Larger cohorts would be necessary, however, to compare the effectiveness of this marker with that of other existing polymorphic markers. Moreover, it should be noted that aberrant methylation of the *RASSF1A* gene promoter can also be found in certain malignant conditions. Therefore, for patients with a known history of cancer, alternative fetal DNA markers may be necessary. In the context of a pregnant woman without a known history of cancer, however, it is unlikely that the plasma methylated *RASSF1A* sequences would be derived from a concurrent occult malignancy rather than from the placenta. We envision that, due to its applicability to all pregnancies and the methodologic simplicity of the assay, this new marker will be an attractive alternative to existing sex-specific and polymorphic markers as positive controls for fetal DNA detection in noninvasive prenatal diagnostic procedures using plasma DNA.

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